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PRECLINICAL STUDY

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Abstract *Purpose* The 70-gene prognosis signature (van't Veer et al., Nature 415(6871):530–536, 2002) may improve the selection of lymph node-negative breast cancer patients for adjuvant systemic therapy. Optimal validation of prognostic classifiers is of great importance and we therefore wished to evaluate the prognostic value of the 70-gene prognosis signature in a series of relatively recently diagnosed lymph node negative breast cancer patients. *Methods* We evaluated the 70-gene prognosis signature in an independent representative series of patients with invasive breast cancer ($N = 123$; <55 years; pT1-2N0; diagnosed between 1996 and 1999; median follow-up 5.8 years) by classifying these patients as having a good or poor prognosis signature. In addition, we updated the follow-up of the node-negative patients of the previously published validation-series (Van de Vijver

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et al., N Engl J Med 347(25):1999–2009, 2002; $N = 151$; median follow-up 10.2 years). The prognostic value of the 70-gene prognosis signature was compared with that of four commonly used clinicopathological risk indexes. The endpoints were distant metastasis (as first event) free percentage (DMFP) and overall survival (OS). *Results* The 5-year OS was 82 ± 5% in poor (48%) and 97 ± 2% in good prognosis signature (52%) patients (HR 3.4; 95% CI 1.2–9.6; $P = 0.021$). The 5-years DMFP was 78 ± 6% in poor and 98 ± 2% in good prognosis signature patients (HR 5.7; 95% CI 1.6–20; $P = 0.007$). In the updated series ($N = 151$; 60% poor vs. 40% good), the 10-year OS was 51 ± 5% and 94 ± 3% (HR 10.7; 95% CI 3.9–30; $P < 0.01$), respectively. The DMFP was 50 ± 6% in poor and 86 ± 5% in good prognosis signature patients (HR 5.5; 95% CI 2.5–12; $P < 0.01$). In multivariate analysis, the prognosis signature was a strong independent prognostic factor in both series, outperforming the clinicopathological risk indexes. *Conclusion* The 70-gene prognosis signature is also an independent prognostic factor in node-negative breast cancer patients for women diagnosed in recent years.

Keywords Gene expression profile · Prognosis signature · Breast cancer · Microarray

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Introduction

Adjuvant systemic treatment substantially improves distant metastasis-free survival and overall survival in lymph node-negative breast cancer patients [1]. It is generally agreed upon that patients with poor prognosis benefit most from this treatment [1, 2]. However, it has a wide range of acute and long-term toxicities. Using traditional risk indexes it has been estimated that 33 patients are treated to save one life [3]. Therefore, an accurate selection of patients who will benefit from adjuvant systemic treatment is essential.

The main prognostic factors in breast cancer are axillary lymph node involvement, age, tumour diameter and histological grade [4]. Clinicopathological risk assessment is based on these factors and is used to guide decisions on adjuvant systemic treatment. A large number of potential prognostic factors have been investigated to predict disease outcome. Even the strongest prognostic factors (e.g. lymph node status, tumour diameter and histological grade) are moderately accurate in classifying breast tumours according to their clinical behaviour. The Oxford Overviews of systemic treatments demonstrate that a significant proportion of patients are long-term survivors without adjuvant systemic therapy [1, 5, 6]. Furthermore, there are patients that will develop metastatic disease despite this treatment.

Several research groups have recently used gene expression profiling to define subgroups of tumours associated with good or poor outcome [7–21]. One of these gene expression profiles predicting breast cancer recurrence is the 70-gene prognosis signature [10]. This prognosis signature potentially is a more powerful prognostic factor for distant metastases than current used clinicalopathological factors in node-negative breast cancer patients. Van de Vijver et al. validated this signature in a series of 295 breast cancer patients [11]. Buyse et al. performed a second international validation in 302 patients [19].

To obtain a reliable estimate of the prognostic value of the 70-gene prognosis signature, validation in several series is essential. The three objectives of this study therefore were: (I) to assess the prognostic value of this prognosis signature in a relatively recently diagnosed, representative node-negative breast cancer patient series; (II) to evaluate its prognostic value in the initial validation-series after expanding the follow-up [11]; and (III) to compare its prognostic value with commonly used clinicopathological risk indexes.

Methods

Patients, tumours and histopathology

Fresh frozen tumour samples (stored at minus 70°C) and clinical data were collected from a consecutive patient

series treated at two Dutch centres, the Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital (NKI; Amsterdam) and the Reinier de Graaf Hospital (RdGG; Delft) between 1996 and 1999. Patients included (<55 years) had received adequate local therapy for early stage breast cancer, defined as node-negative, tumour diameter pT1-2. Patients with prior malignancies (except basal cell carcinomas and cervical dysplasia) or bilateral breast cancers were excluded.

Hereafter this validation series is referred to as 'the NKI-RdGG validation series'. Institutional approval from the medical-ethical committee of the NKI was obtained for conduct of this study.

Collection of clinical and pathological data, as well as central review of paraffin-embedded tumour samples was performed at the NKI blinded to the prognosis signature. Histological grade according to the Elston & Ellis method [22], oestrogen receptor and progesterone receptor (assessed by immunohistochemistry) were determined by two experienced breast pathologists (JW and MJvdV). In case of discordance between the original pathological examination and central review with regard to grade, the examination was performed again by a panel of three experienced breast pathologists (JW, MJvdV, JLP) who then agreed on a final grade. The oestrogen and progesterone receptor were considered positive if 10% or more of tumour cells stained positive using an immunohistochemical assay.

RNA extraction & microarray analysis

A patient's (good or poor) 70-gene prognosis signature was determined using frozen samples. Frozen sections were stained with H&E and analyzed by an experienced breast pathologist (MJvdV). Eligible samples had to contain at least 50% tumour cells. Thirty-seven patients (24 NKI and 13 RdGG patients) were excluded because the percentage of tumour cells was insufficient; however, the clinical and pathological characteristics of the excluded patients were not significantly different from those in the study population (data not shown).

Details of RNA isolation, microarray analysis and correlation of microarray data with the prognosis signature have previously been described [10, 11, 23]. Microarray analysis for obtaining the 70-gene prognosis signature (MammaPrint®) was performed at Agendia laboratories, a spin-off company of the NKI, blinded to clinical outcome data. Agendia's 'MammaPrint diagnostic service' is cleared by the Food and Drug Administration as a medical device and is ISO-17025 accredited, utilizing a custom designed array chip "MammaPrint®". This array chip assesses the mRNA expression of the 70 genes in triplicate using the Agilent oligonucleotide microarray platform.

Established clinical risk classifications indexes

Hereafter clinicopathological risk is referred to as 'clinical risk', which was based on data of centrally reviewed tumour samples. Clinicopathological risk indexes included: St Gallen guidelines [24–28], Nottingham Prognostic Index [29–31], Dutch CBO guidelines 2004-available at www.oncoline.nl- [32, 33] and Adjuvant! Online-available at www.adjuvantonline.com [34–36]. In this study, a moderate or high clinical risk was an indication for adjuvant systemic treatment.

According to St Gallen guidelines, a low clinical risk was defined as oestrogen and/or progesterone positive and all of the following features: tumour size smaller or equal to 2 cm, grade 1 (Elston & Ellis), and age equal or above 35 years [24–28]. All others were considered as moderate or high risk.

The Nottingham Prognostic Index (NPI) computes a score using the following algorithm: 0.2 * size (cm) + grade + nodal status [29–31].

The Adjuvant! Online Software calculates a 10-year survival probability based on the patient's age, tumour size, histological tumour grade, oestrogen receptor status and nodal status [34–36]. A low clinical risk was defined as patients with a 10-years survival probability of at least 90%.

According to the Dutch CBO guidelines 2004, a low clinical risk was defined as being above the age of 35 years and having a tumour of grade 1 with size less than 30 mm, grade 2 and size less than 20 mm, or grade 3 with size 10 mm or less [32, 33].

Each of the clinical risk indexes was combined with the prognosis signature, resulting in groups of patients as clinical low risk-good prognosis signature, clinical low risk-poor prognosis signature, clinical high risk-good prognosis signature, and clinical high risk-poor prognosis signature patients.

Statistical analysis

Calculations were performed using the S+ (version 6.2) statistical package and SPSS (version 14.0 for Windows). The differences in patients and tumour characteristics between the 70-gene prognosis signature good and poor patients were tested using the Pearson Chi-Square test. In case of ordinal variables (year of diagnosis, age, and histological grade) with more than two groups, we tested for trends (using Cochran-Armitage test). The level of agreement between the guidelines and the prescription of adjuvant systemic treatment was expressed by means of a Cohen's kappa. A kappa of one indicates perfect agreement, where a kappa of zero indicates no agreement. The two main endpoints were: time from surgery to distant

metastasis as first event, which was the endpoint used to identify the 70-gene signature [10]; and overall survival, defined as time from surgery to death.

In the analysis of distant metastasis, patients whose first failure was distant metastasis were counted as failures; all other patients were censored at the date of their last follow-up, death, contralateral breast cancer, other second primary or loco-regional recurrence. *P*-values have not been corrected for multiple comparisons.

Metastasis as first event

Unadjusted metastasis-free curves were drawn using the method of Kaplan–Meier [37] and compared using the Wald test based on Cox's Proportional Hazard regression analysis [38]. Values are expressed as percentages ±SE, calculated according to the method of Tsiatis [39].

Proportional hazard regression analysis was also used to adjust the association between the prognosis signature and metastasis for other variables. For this adjustment we chose on a-priori grounds the variables used in the four mentioned clinical risk assessment methods for the prediction of recurrence. These are age, tumour diameter, histological grade and oestrogen receptor status. We added adjuvant chemo- and hormonal treatment and clinical risk as well. Categorical variables (centre, year of surgery, tumour diameter, Grade and clinical risk) were used as factors, using the first category as reference.

Logistic regression analysis was performed to estimate the additional value of the 70-gene prognosis signature to traditional prognostic clinicopathological risk factors (age, tumour size, histologic grade, oestrogen receptor, progesterone receptor, and HER2-receptor). The traditional prognostic model, based on the above mentioned prognostic factors, was compared to a new model also including the prognosis signature. Time dependent receiver operator characteristic (ROC-) curves were computed for both distant metastasis as first event and overall survival by using the 70-gene prognosis signature outcome and the above mentioned prognostic factors.

Update follow-up initial validation series

The follow-up of the 151 node-negative patients of the initial validation series was updated until January 2005, blinded to the 70-gene prognosis signature and clinical risks [11]. The same statistical analyses were performed as described above. In the logistic regression analysis only age, tumour size, histologic grade, and oestrogen receptor were used as prognostic parameter in the traditional model while progesterone receptor and HER2 status were not available for this dataset.

Results

NKI-RdGG validation series

For 123 node-negative invasive breast cancer patients (NKI-RdGG validation series) a 70-gene prognosis profile was obtained. Patient characteristics are summarized in Table 1. Median follow-up was 5.8 years (range: 0.1–9.0), mean age was 47 years (range: 27–55) and the mean tumour diameter was 20 mm (range: 5–50). Adjuvant systemic treatment was administered to 37% (45/123) of the patients; 15% (18/123) chemotherapy, 11% (14/123) endocrine treatment and 11% (13/123) both. During follow-up 30 cancer-related first events occurred [14 distant metastases (47%), 5 local recurrences (17%), 3 regional recurrences (10%), 4 contralateral breast cancers (13%), and 4 second primary cancers (13%; other types of cancer)]. Eighteen patients died (17 breast cancer related and 1 due to another cancer).

Fifty-two percent of the patients had a tumour with a good and 48% with a poor prognosis signature. The poor prognosis signature was associated with larger tumours, a high histological grade, and a negative oestrogen and progesterone receptor; these patients received more often adjuvant systemic treatment (Table 1). In the sub-group of patients that did receive any form of adjuvant systemic treatment (45; 37%), 42% (19/45) had a good and 58% (26/45) a poor prognosis signature (data not shown).

Clinical risk and prognosis signature

According to the Adjuvant! Online, the NPI, the St Gallen guidelines and Dutch CBO guidelines 2004, respectively 76, 51, 87 and 55% of the patients were assessed as moderate or high risk (Table 1). In 38% of the patients (47/123; kappa 0.25) the clinical risk according to the Adjuvant! Online was discordant with the prognosis signature. Six patients (5%) were Adjuvant! Online clinical low risk and had a poor prognosis signature; and 41 patients (33%) were clinical high risk and had a good prognosis signature (Suppl. Table 1). If CBO guidelines 2004, St Gallen guidelines or NPI were used, respectively in 30% (37/123; kappa 0.40), 41% (50/123; 0.21) and 26% (32/123; kappa 0.48) of the patients the risk assessment was discordant with prognosis signature. Figure 1a–d show the Kaplan–Meier curves with distant metastasis as first event and overall survival, respectively, for prognosis signature and clinical risk using Adjuvant! Online for the NKI-RdGG series.

At 5 years, the probability of remaining free of distant metastasis (as first event) was 98% (SE 2%) for good and 78% (SE 6%) for poor prognosis signature patients with an estimated hazard ratio (HR) of 5.7 (95% CI: 1.6–20; $P = 0.007$; Table 2) in the univariate analysis. The 5-year

Table 1 Relations of clinical and pathological variables with 70-gene prognosis signature in the NKI-RdGG validation series and the updated initial validation series

	NKI-RdGG validation series				Updated initial validation series			
	Total *n* (%)	Good *n* (%)	Poor *n* (%)	*P*-value	Total *n* (%)	Good *n* (%)	Poor *n* (%)	*P*-value
Total	123	64 (52%)	59 (48%)	–	151	60 (40%)	91 (60%)	
Hospital				0.15				
RdGG	52 (42%)	31 (60%)	21 (40%)	–	–	–	–	–
NKI	71 (58%)	33 (46%)	38 (54%)	–	–	–	–	–
Age				0.19				0.072
≤35 years	12 (10%)	4 (33%)	8 (67%)	–	13 (9%)	2 (15%)	11 (85%)	–
36–40 years	10 (8%)	7 (70%)	3 (30%)	–	32 (21%)	9 (28%)	23 (72%)	–
41–45 years	20 (16%)	8 (40%)	12 (60%)	–	41 (27%)	21 (51%)	20 (49%)	–
46–50 years	41 (33%)	21 (51%)	20 (49%)	–	51 (34%)	22 (43%)	29 (57%)	–
>50 years	40 (33%)	24 (60%)	16 (40%)	–	14 (9%)	6 (43%)	8 (57%)	–
Type of surgery				0.020				
Breast conserving	89 (72%)	54 (61%)	35 (39%)	–	–	–	–	–
Mastectomy	34 (28%)	10 (29%)	24 (71%)	–	–	–	–	–
Tumour size (pTNM)				0.016				0.032
pT1 (≤20 mm)	76 (62%)	46 (61%)	30 (39%)	–	82 (54%)	39 (48%)	43 (52%)	–
pT2 (21–50 mm)	47 (38%)	18 (38%)	29 (62%)	–	69 (46%)	21 (30%)	48 (70%)	–
Histologic grade				$P < 0.001$				$P < 0.001$
1 (well)	20 (16%)	18 (90%)	2 (10%)	–	34 (23%)	27 (79%)	7 (21%)	–
2 (intermediate)	53 (43%)	35 (66%)	18 (34%)	–	46 (30%)	23 (50%)	23 (50%)	–
3 (poor)	50 (41%)	11 (22%)	39 (78%)	–	71 (47%)	10 (14%)	61 (86%)	–
Oestrogen receptor				$P < 0.001$				$P < 0.001$
Negative	29 (24%)	2 (7%)	27 (93%)	–	42 (28%)	2 (5%)	40 (95%)	–
Positive	94 (76%)	62 (66%)	32 (34%)	–	109 (72%)	58 (53%)	51 (47%)	–
Progesterone receptor				$P < 0.001$				
Negative	39 (32%)	6 (15%)	33 (85%)	–	–	–	–	–
Positive	84 (68%)	58 (69%)	26 (31%)	–	–	–	–	–
HER2-receptor				0.067				
Negative	113 (93%)	61 (54%)	52 (46%)		–	–	–	–
Positive	9 (7%)	2 (22%)	7 (78%)		–	–	–	–
Missing: 1 (1%)								
Adjuvant systemic treatment				$P < 0.001$				0.11
None	78 (63%)	45 (58%)	33 (42%)	–	141 (93%)	57 (40%)	84 (60%)	–
Chemotherapy	18 (15%)	1 (6%)	17 (94%)	–	4 (3%)	–	4 (100%)	–
Endocrine therapy	14 (11%)	9 (64%)	5 (36%)	–	4 (3%)	1 (25%)	3 (75%)	–
Both	13 (11%)	9 (69%)	4 (31%)	–	2 (1%)	2 (100%)	–	–
St. Gallen guidelines				$P < 0.001$				$P < 0.001$
Low risk	16 (13%)	15 (94%)	1 (6%)	–	19 (13%)	15 (79%)	4 (21%)	–
Intermediate/high risk	107 (87%)	49 (46%)	58 (54%)	–	132 (87%)	45 (34%)	87 (66%)	–
Nottingham Prognostic Index				$P < 0.001$				$P < 0.001$
Low risk	60 (49%)	46 (77%)	14 (23%)	–	67 (44%)	44 (66%)	23 (34%)	–
Moderate/high risk	63 (51%)	18 (29%)	45 (71%)	–	84 (56%)	16 (19%)	68 (81%)	–
Dutch CBO guidelines 2004				$P < 0.001$				$P < 0.001$
Low risk	55 (45%)	41 (75%)	14 (25%)	–	56 (37%)	36 (64%)	20 (36%)	–
High risk	68 (55%)	23 (34%)	45 (66%)	–	95 (63%)	24 (25%)	71 (75%)	–

Table 1 continued

	NKI-RdGG validation series				Updated initial validation series			
	Total *n* (%)	Good *n* (%)	Poor *n* (%)	*P*-value	Total *n* (%)	Good *n* (%)	Poor *n* (%)	*P*-value
Adjuvant! Online				0.001				$P < 0.001$
Low risk	29 (24%)	23 (79%)	6 (21%)	–	37 (25%)	23 (62%)	14 (38%)	–
High risk	94 (76%)	41 (44%)	53 (56%)	–	114 (75%)	37 (32%)	77 (68%)	–

RdGG, Reinier de Graaf Hospital, Delft, The Netherlands; NKI, Netherlands Cancer Institute, Amsterdam, The Netherlands. NPI, Nottingham Prognostic Index

Note: percentages in tables and texts may not add up to 100% or add up over 100% due to rounding off

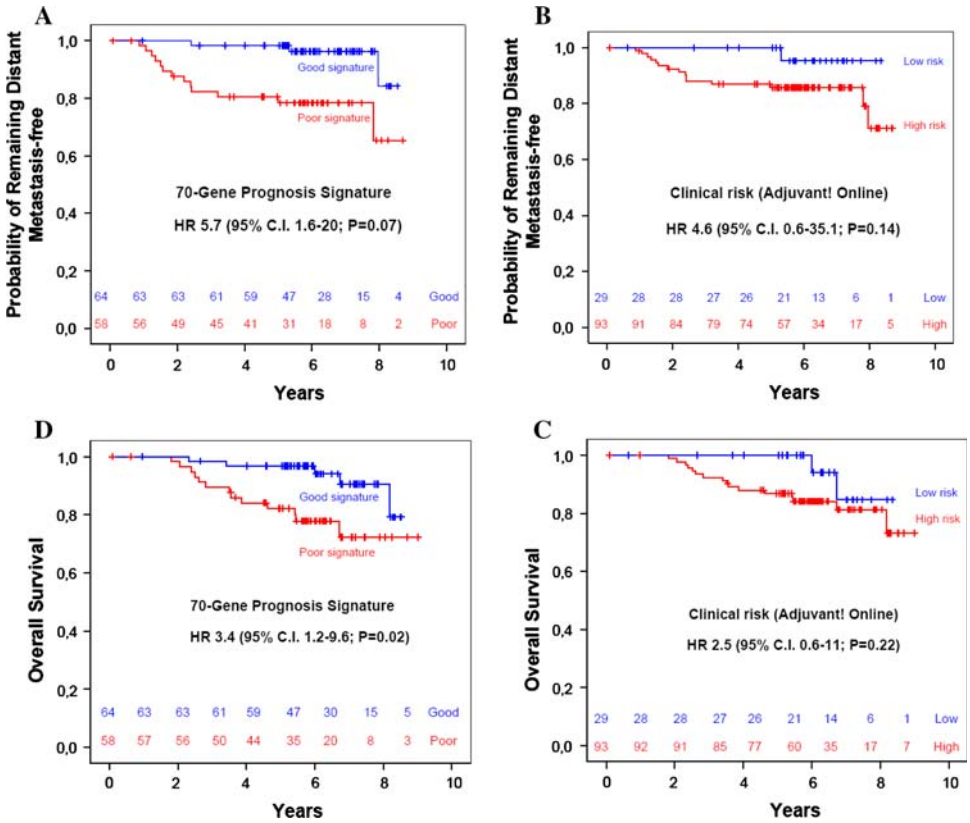

Fig. 1 Kaplan–Meier Curves of metastasis as first event (**a, b**) and overall survival (**c, d**) of the 70-gene prognosis signature (good prognosis signature versus poor prognosis signature) (**a, c**) and Adjuvant! Online (low risk versus high risk) (**b, d**) in the NKI-RdGG validation series

overall survival probability was 97% (SE 2%) for good and 82% (SE 5%) for poor prognosis signature patients with an estimated HR of 3.4 (95% CI: 1.2–9.6; $P = 0.021$). In this series none of the four clinical risk indexes did have prognostic power (Table 2) for distant metastasis as first event or overall survival; and neither did the combined clinical risk-prognosis signatures for each of the clinical risk indexes (data not shown).

Multivariate analysis with clinical risk indexes

The prognostic value of the prognosis signature remained strongly significant in the multivariate analysis for distant metastasis as first event and overall survival when adjusted for the four clinical risk indexes. Its HR with distant metastasis as first event in the univariate analysis was 5.7 and ranged in the multivariate analysis from 4.8 to 5.8 dependent on what index was used (Table 3A). For overall survival the univariate HR was 3.4 and in the multivariate analysis it ranged from 2.7 to 3.3. If the performance of these four clinical risk indexes was adjusted for the prognosis signature, none of the clinical risk indexes were found to have an independent association with distant metastasis as first event or with overall survival (Table 3B).

Logistic regression analysis and ROC curves

In the logistic regression analysis, comparing the traditional prognostic model (based on age, tumour size,

Table 2 Results of univariate analysis with distant metastasis as first event and overall survival for patients of the NKI-RdGG validation series

Variable	Unit	Distant metastasis as first event			Overall survival		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
70-gene prognosis signature	Poor vs. good	5.7	1.6–20	0.007	3.4	1.2–9.6	0.021
Institute	RdGG vs. NKI	0.81	0.30–2.2	0.68	1.2	0.46–3.3	0.69
Year of surgery	1997 vs. 1996	0.37	0.08–1.8	0.15	0.35	0.07–1.6	0.32
	1998 vs. 1996	0.25	0.05–1.2		0.56	0.17–1.9	
	1999 vs. 1996	0.29	0.08–1.1		0.35	0.09–1.3	
Surgery	Ablation vs. BCT	2.3	0.86–6.2	0.097	3.6	1.4–9.2	0.007
Age	Per year	1.0	0.93–1.1	0.99	0.93	0.94–1.1	0.93
Diameter	pT2 vs. pT1	1.0	0.37–2.8	0.99	0.89	0.33–2.4	0.83
Histologic grade	Moderate vs. good	0.86	0.16–4.7	0.18	2.5	0.26–19	0.057
	Poor vs. good	2.4	0.52–11		6.1	0.80–47	
Oestrogen receptor	Pos. vs. neg.	0.46	0.17–1.2	0.14	0.40	0.15–1.0	0.057
Progesterone receptor	Pos. vs. neg.	0.56	0.21–1.5	0.25	0.52	0.21–1.3	0.18
Adjuvant systemic treatment	None vs. any adjuvant systemic treatment[a]	0.64	0.21–2.0	0.44	0.80	0.28–2.3	0.67
St Gallen guidelines	Intermediate/high vs. low risk	2.5	0.34–19	0.37	3.0	0.40–22	0.29
NPI	Moderate vs. low risk	2.2	0.78–6.5	0.14	2.8	0.99–7.8	0.053
CBO guidelines 2004	High vs. low risk	1.8	0.64–5.3	0.26	2.3	0.84–6.6	0.11
Adjuvant! Online	High vs. low risk	4.6	0.61–35	0.14	2.5	0.59–11	0.22
Combined clinical-prognosis signature risk[b]	Low-poor vs. low-good	0.00	0.00–∞	0.049	0.00	0.00–∞	0.10
	High-good vs. low-good	1.1	0.10–12		0.83	0.14–5.0	
	High-poor vs. low-good	6.3	0.83–49		3.3	0.75–15	

[a] Adjuvant systemic treatment may consist of chemotherapy, endocrine treatment or both

[b] Combined clinical and prognosis signature risk using Adjuvant! Online and 70-gene prognosis signature. Risk stated before dash is clinical risk. After the dash the prognosis signature is stated

Note: percentages in tables and texts may not add up to 100% or add up over 100% due to rounding off

RdGG, Reinier de Graaf Hospital in Delft, the Netherlands; NKI, Netherlands Cancer Institute in Amsterdam, the Netherlands. NPI, Nottingham Prognostic Index. BCT, Breast conserving treatment

histologic grade, oestrogen receptor, progesterone receptor, and HER2 status) in the presence versus the absence of the prognosis signature, the change in log likelihood for distant metastasis was 5.2 ($P = 0.023$). The area under the ROC curve (AUC; Fig. 2) for the predicted probability in the absence and presence of the prognosis signature was 0.66 (95% CI 0.50–0.82) and 0.75 (95% CI 0.61–0.89), respectively. The AUC for the prognosis signature alone was 0.69 (95% CI 0.56–0.82). Although the confidence intervals overlap there appears to be additional value for the model in which the prognosis signature is combined with the traditional prognostic factors, as shown by the larger AUC. The ROC curve for overall survival showed that the prognosis signature did not add value to the prognostic model as the AUC's did not differ substantially and 95% confidence intervals overlap almost completely for the different models (Fig. 2). The AUC of the predicted probability of the model in the absence and presence of the prognosis signature were, respectively 0.69 (95% CI 0.56–0.82) and 0.69 (95% CI 0.55–0.84); the AUC for the prognosis signature was 0.64 (95% CI 0.51–0.77).

Updated initial validation series

By updating the median follow-up from 7.3 years to 10.2 years (range: 0.7–21.3), for the 151 node-negative patients of the initial validation series [11] 8 additional deaths occurred. During the 10.2 years of follow-up 74 breast cancer-related first events and 51 deaths (48 breast cancer related and 3 deaths due to other causes) occurred. The first events were: 48 distant metastases (65%), 8 local recurrences (11%), 6 regional recurrences (8%), 10 contralateral breast cancers (14%), and 2 second primary breast cancers (3%).

Poor prognosis signature patients in this series were more often high risk patients according to the clinical indexes mentioned above than the good prognosis signature patients (Table 1).

Table 3 Results of multivariate analysis with distant metastasis as first event and overall survival. Performance of prognosis signature adjusted for prognostic clinical risks in the NKI-RdGG validation series (A) and updated initial validation series (C); and performance of other prognostic clinical risk indexes adjusted for prognosis signature in the NKI-RdGG validation series (B) and updated initial validation series (D)

Adjusted for	Performance of 70-gene prognosis signature (poor vs. good)					
	Distant metastasis as first event			Overall survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
(A) *NKI-RdGG validation series*						
Adjuvant! Online	4.8	1.3–17	0.018	3.0	1.0–8.9	0.044
St. Gallen guidelines	5.8	1.5–22	0.011	3.1	1.0–9.2	0.043
Nottingham Prognostic Index	5.4	1.4–21	0.015	2.7	0.87–8.1	0.086
Dutch CBO guidelines 2004	5.6	1.5–21	0.010	2.9	0.98–8.6	0.055
Predictor	Performance of predictor (high vs. low risk) adjusted for 70-gene prognosis signature					
	Distant metastasis as first event			Overall survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
(B) *NKI-RdGG validation series*						
Adjuvant! Online	2.5	0.32–20	0.38	1.65	0.36–7.7	0.52
St. Gallen guidelines	0.95	0.11–8.4	0.97	1.64	0.20–14	0.65
Nottingham Prognostic Index	1.1	0.36–3.5	0.83	1.90	0.62–5.8	0.26
Dutch CBO guidelines 2004	1.0	0.34–3.2	0.94	1.66	0.56–4.9	0.36
Adjusted for	Performance of 70-gene prognosis signature (poor vs. good)					
	Distant metastasis as first event			Overall survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
(C) *Updated initial validation series*						
Adjuvant! Online	5.3	2.4–12	$P < 0.001$	9.6	3.4–27	$P < 0.001$
St. Gallen guidelines	5.2	2.3–12	$P < 0.001$	9.9	3.5–28	$P < 0.001$
NPI	4.3	1.8–10	0.001	8.5	2.9–25	$P < 0.001$
CBO guidelines 2004	4.9	2.1–11	$P < 0.001$	8.3	2.9–24	$P < 0.001$
Predictor	Performance of predictor (high vs. low risk) adjusted for 70-gene prognosis signature					
	Distant metastasis as first event			Overall survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
(D) *Updated initial validation series*						
Adjuvant! Online	1.2	0.58–2.5	0.61	1.7	0.72–4.1	0.22
St. Gallen guidelines	1.4	0.41–4.6	0.61	1.6	0.38–6.8	0.53
NPI	1.9	0.94–3.7	0.073	1.7	0.86–3.3	0.13
CBO guidelines 2004	1.5	0.74–2.9	0.27	2.2	1.0–4.8	0.044

HR: hazard ratio; 95% CI: 95% confidence interval

Using the Adjuvant! Online, NPI, the St Gallen guidelines, and CBO guidelines 2004, respectively 75, 56, 87, and 63% were at moderate or high risk for recurrence. In 34% of the patients (51/151; kappa 0.24), the clinical risk according to the Adjuvant! Online was discordant with the prognosis signature. Fourteen patients (9%) were Adjuvant! Online clinical low risk and had a poor prognosis signature; and vice versa, 37 patients (25%) were clinical high risk and had a good prognosis signature. If CBO guidelines 2004, St Gallen guidelines or NPI were used, respectively 29%, (44/151; kappa 0.38), 32% (49/151; kappa 0.23) and 26% (39/151; kappa 0.47) was discordant with the prognosis signature.

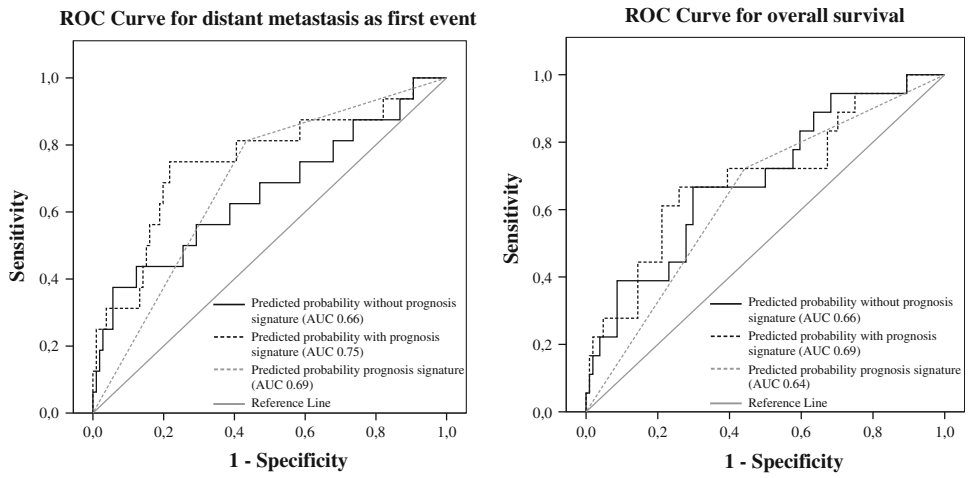

Fig. 2 Receiver operating characteristic (ROC-) curves of prognosis signature, prognostic factors and both for the NKI-RdGG validation series. Prognostic factors used in model were age, tumour size, histologic grade, oestrogen receptor, progesterone receptor, and HER2 status for distant metastasis as first event (left) and overall survival (right). *AUC = area under the curve*

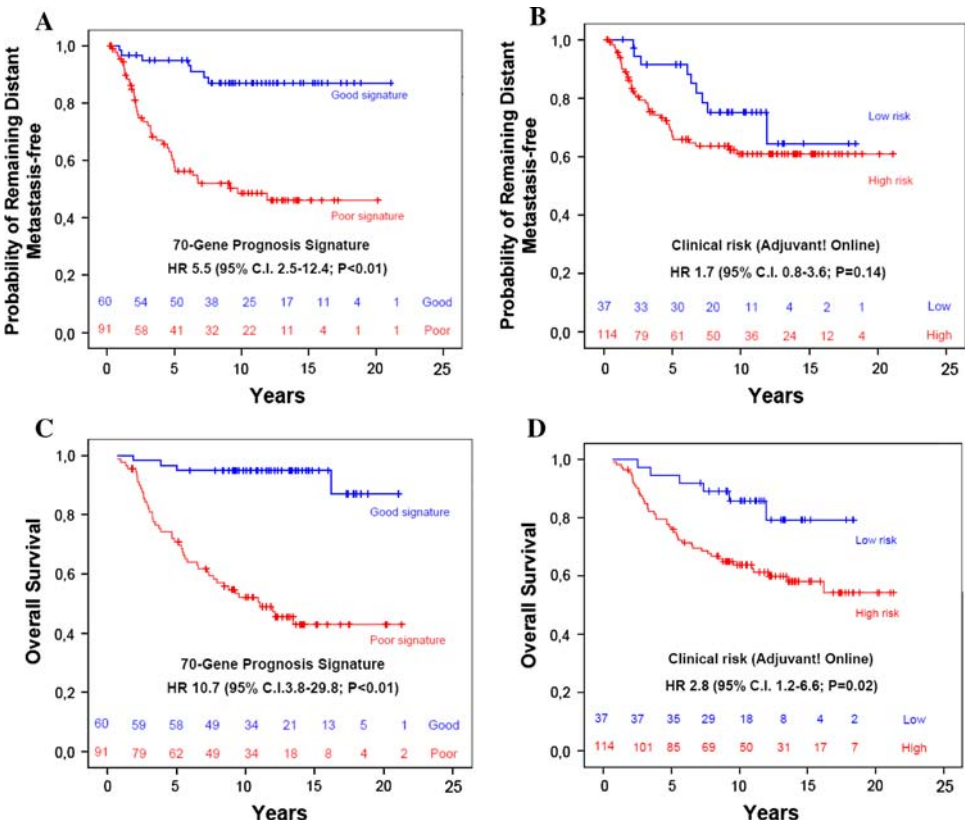

Fig. 3 Kaplan–Meier curves of metastasis as first event (**a, b**) and overall survival (**c, d**) of the 70-gene prognosis signature (good prognosis signature versus poor prognosis signature) (**a, c**) and Adjuvant! Online (low risk versus high risk) (**b, d**) in the updated initial validation series

Figure 3a–d show the Kaplan–Meier curves of distant metastasis as first event and overall survival for the prognosis signature and clinical risk index (Adjuvant! Online). At 10 years, the distant metastasis (as first event) free survival was 86% (SE 5%) for good and 50% (SE 6%) for poor prognosis signature patients with an estimated HR of 5.5 (95% CI: 2.5–12, $P < 0.001$; univariate analysis; Table 4). The estimated 10-year overall survival was 94% (SE 3%) in good and 51% (SE 5%) in poor prognosis signature patients with an estimated HR of 10.7 (95% CI: 3.9–30; $P < 0.001$; univariate analysis).

In this updated initial validation series the NPI clinical risk had prognostic power for both distant metastasis as first event and overall survival (Table 4). The clinical risk using Adjuvant! Online (90%) and Dutch CBO guidelines 2004 only had prognostic value for overall survival. The combined clinical risk- prognosis signatures (for each of the clinical risk indexes) had a performance in between the clinical risk indexes and the prognosis signature (Table 4 and data not shown).

The performance of the prognosis signature adjusted for the four clinical risk indexes using distant metastasis as

Table 4 Results of univariate analysis with distant metastasis as first event and overall survival in the updated initial validation series [11]

Variable	Unit	Distant metastasis as first event			Overall survival		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
70-Gene prognosis signature	Poor vs. good	5.5	2.5–12	$P < 0.001$	10.7	3.9–30	$P < 0.001$
Age	Per year	0.96	0.92–1.0	0.18	0.96	0.91–1.0	0.091
Diameter	pT2 vs. pT1	1.9	1.1–3.3	0.014	2.0	1.1–3.5	0.015
Histologic grade	Moderate vs. good	3.1	1.0–9.2	0.040	5.9	1.3–26	0.010
	Poor vs. good	5.4	1.9–15		11	2.7–47	
Oestrogen receptor	Pos. vs. Neg.	0.58	0.33–1.0	0.058	0.37	0.21–0.65	0.010
Adjuvant systemic treatment	None vs. any adjuvant systemic treatment[a]	1.2	0.72–1.9	0.55	1.2	0.78–2.0	0.36
St Gallen guidelines	Intermediate/high vs. low risk	2.6	0.80–8.3	0.11	4.1	0.99–17	0.052
NPI	Moderate vs. low risk	3.1	1.6–5.9	$P < 0.001$	3.4	1.8–6.6	$P < 0.001$
CBO guidelines 2004	High vs. low risk	2.4	1.2–4.5	0.011	3.9	1.9–8.4	$P < 0.001$
Adjuvant! Online	High vs. low risk	1.7	0.84–3.6	0.14	2.8	1.2–6.6	0.017
Combined clinical-prognosis signature risk[b]	Low-poor vs. low-good	3.9	0.99–16	$P < 0.010$	∞	0.00–∞	$P < 0.004$
	High-good vs. low-good	0.9	0.19–3.9		∞	0.00–∞	
	High-poor vs. low-good	5.3	1.6–17		∞	0.00–∞	

[a] Adjuvant systemic treatment may consist of chemotherapy, endocrine treatment or both

[b] Combined clinical and prognosis signature risk using Adjuvant! Online and 70-gene prognosis signature. Risk stated before dash is clinical risk. After the dash the prognosis signature is stated

Note: percentages in tables and texts may not add up to 100% or add up over 100% due to rounding off

NPI, Nottingham Prognostic Index

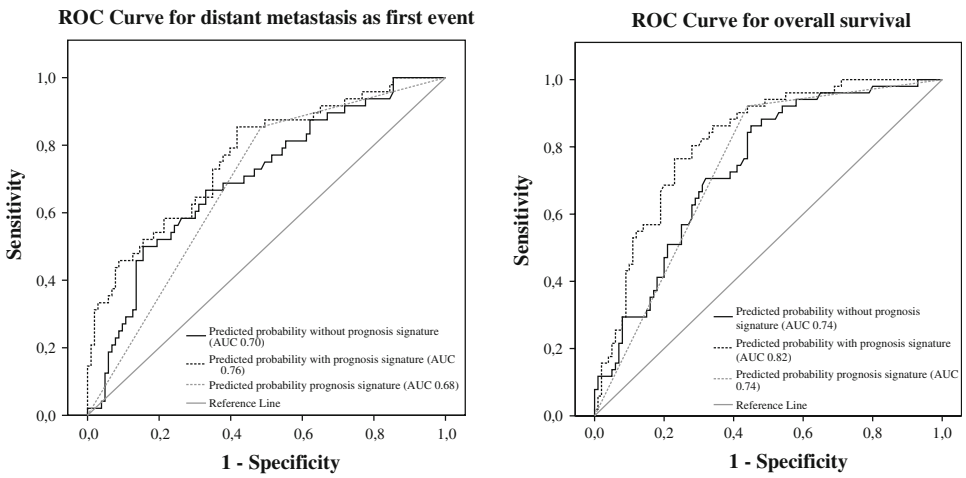

Fig. 4 Receiver operating characteristic (ROC) curves of prognosis signature, prognostic factors and both for the updated initial validation series. Prognostic factors used in model were age, tumour size, histologic grade, and oestrogen receptor for distant metastasis as first event (left) and overall survival. In both curves the diagonal segments are produced by ties (right). *AUC = area under the curve*

first event or overall survival remained stable (Table 3C). If the performance of these clinical risk indexes was adjusted for the prognosis signature, none of the clinical risk indexes retained an independent association with distant metastasis as first event or overall survival (with the exception of the CBO guidelines 2004 regarding overall survival; Table 3D).

In the logistic regression analysis the traditional prognostic model in the presence versus the absence of the prognosis signature, the change in log likelihood for distant metastasis was 15.8 ($P < 0.001$). The AUC of the ROC curve (Fig. 4) for the predicted probability in the absence and presence of the prognosis signature was, respectively 0.70 (95% CI 0.61–0.79) and 0.76 (95% CI 0.68–0.85). The AUC for the prognosis signature alone was 0.68 (95% CI 0.60–0.77). Although the confidence intervals overlap there appears to be additional value for the model in which the prognosis signature was combined with the traditional prognostic factors as the AUC was larger. The change in log likelihood for overall survival was 19.7 ($P < 0.001$) and showed that the prognosis signature adds additional value to the prognostic model. The AUC for overall

survival of the predicted probability of the model in the absence and presence of the prognosis signature were, respectively 0.74 (95% CI 0.66–0.82) and 0.82 (95% CI 0.75–0.89); the AUC for the prognosis signature is 0.74 (95% CI 0.66–0.82).

Discussion

In the NKI-RdGG validation-series of relatively recently diagnosed breast cancer patients as well as in the updated initial validation series, the 70-gene prognosis signature was a prognostic factor for distant metastasis as first event (respectively HR 5.7 and 5.5) and overall survival (respectively HR 3.4 and 10.7) in the univariate analysis. After adjustment for commonly used clinical risk indexes, the prognosis signature remained an independent prognostic factor. In contrast, if the clinical risk indexes were adjusted for the prognosis signature, their prognostic value was lost. The logistic regression analysis suggests that the prognosis signature would have additional value in clinical practice if the traditional prognostic factors would be combined with the prognosis signature. The confidence intervals did overlap, probably because of small sample size in both series and relatively short follow up in the NKI-RdGG validation series.

Buyse et al. had previously confirmed the prognostic value of the 70-gene prognosis signature in the first international and independent validation study in 2006 (TRANSBIG validation; $N = 302$; median follow-up: 13.6 years; Suppl. Table 3) [19]. These patients had been selected for not having received any form of adjuvant systemic treatment, in order to be able to study the true prognostic value not confounded by adjuvant systemic treatment and were diagnosed between 1990 and 1998. The HR of the prognosis signature for distant metastasis free survival was 2.3 ($P = 0.002$; univariate) and for overall survival it was 2.8 ($P < 0.001$). Remarkably, in this series classical clinicopathological factors did not have prognostic value for distant metastases as first event and overall survival (univariate analysis); the only exception was the oestrogen receptor (respectively, HR 2.2 (95% CI 1.4–3.5; $P = 0.001$) and HR 2.4 (95% CI 1.5–3.7; $P < 0.001$). In our updated initial validation series with comparable follow-up these factors did perform as prognostic factors for both distant metastasis and overall survival. The classical clinicopathological factors did not have prognostic value in the NKI-RdGG validation series. This was probably due to the small number of events in this series. In this series 37% of the patients received some form of adjuvant systemic treatment. The proportion of poor signature patients (58%) in this treated group was slightly larger than the good prognosis signature patients (42%; $P < 0.01$). Interestingly, if we assume that the proportional risk reduction of adjuvant systemic treatment is similar for both poor and good prognosis signature patients, then the prognostic value of the prognosis signature would even have been higher with only untreated patients.

In this validation study only 7 and 5% of the patients with oestrogen-receptor-negative tumours in the NKI-RdGG and updated initial validation series, respectively, had a good prognosis signature. The prognosis signature has less discriminative value regarding prognosis in this subgroup. Additional prognostic tests might be needed for these patients.

In breast cancer, two other prognostic gene expression profiles have been reported, the 76-gene signature [17, 18, 20] and the 21-gene recurrence score (Oncotype DX® assay) [14, 15, 21]. The 76-gene signature identified node-negative breast cancer patients at high risk of recurrence. The 21-gene recurrence score quantified the likelihood of distant recurrence in tamoxifen-treated patients with node-negative, oestrogen-receptor positive breast cancer. A recent comparison of the 70-gene signature and an estimation of the 21-recurrence score using microarray data suggests that these two prognostic signatures identify partly overlapping series of patients [40].

For these promising gene expression profiles, prospective studies will ultimately show that indeed a survival benefit can be achieved while avoiding unnecessary adjuvant systemic treatment. The 70-gene prognosis signature has been used in the Dutch RASTER-study (Netherlands Cancer Institute in collaboration with the Dutch Health Care Insurance Board) [41] and is currently used in the international MINDACT-trial (TRANSBIG consortium in collaboration with the European Organisation for Research and Treatment of Cancer) [42].

Conclusion

In conclusion, the results of this representative independent validation of the 70-gene signature are in agreement with our previous findings [11] and those of the TRANSBIG validation study [19] now also including more recently diagnosed patients. It provides evidence that this prognosis signature is an independent prognostic factor in node-negative breast cancer and outperforms current traditional clinical indexes. Using this prognosis signature in addition to the traditional clinical indexes may improve the selection of patients benefiting from adjuvant systemic treatment.

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Competing interest statement Dr. L.J. Van 't Veer, Dr. M.J. Van de Vijver and A.A.M. Hart are named inventors on a patent application for the 70-gene signature used in this study. Dr. Van't Veer report being shareholder in and (part time) employed by Agendia, the commercial company that markets the 70-gene signature as MammaPrint®. A.N. Floore and A.M. Glas report being employed by Agendia.

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